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14. ABSTRACT

This projects seeks to establish a key role for cellular sulfonation enzymes in the metastatic progression of breast cancer. To this end we have accomplished the following tasks in this first year of support:

1. Creation Tetracycline inducible WT Snail and mutant Snail vectors for expression in cell lines,
2. Creation of stable cell lines in MCF10A cells and 67NR cells via a lentiviral expression system.
3. Demonstration MCF-10A cells und ergo dramatic morphologic EMT in the presence of W T Snail expression, with the downregulation of the Cell adhesion molecule E-cadherin and the upregulation of the mesenchymal markers vimentin and fibronectin.
4. Characterization of antibodies to PAPSS1 and PAPSS2 enzymes.
5. Demonstration that both enzymes are expresse d in Human breast cancer cell lines a with a positive correlation to Snail levels.
6. Derivation and characterization of shRNA knockdown vectors for PAPSS1 and PAPSS2.

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INTRODUCTION:

Background: The Snail transcription factor is a strong inducer of epithelial-mesenchymal transition (EMT) in normal embryonic development and tumor metastasis. In mice, Snail is spontaneously up-regulated during tumor recurrence in the mammary gland and high Snail expression strongly predicts decreased relapse-free survival in women with breast cancer, suggesting that Snail may play a critical role in tumor recurrence. All evidence indicates that Snail induces EMT and metastasis at least partially due to repression of *E-cadherin* and other genes encoding cell junction proteins, thereby altering cell adhesion. Thus, previous studies have focused on defining Snail-associated repression complexes and identifying genes repressed by Snail. Interestingly, our preliminary studies provided with the original proposal demonstrated that expression of the *PAPSS2* gene, which encodes the enzyme 3 -phosphoadenosinse 5-phosphosulfate synthase 2, a rate-limiting component of the sulfonaiton pathway, was induced by Snail in breast cancer cells. Depletion of PAPSS2 expression in highly metastatic breast cancer cells led to acquisition of the epithelial phenotype. PAPSS2 catalyzes the synthesis of 3 phosphoadenosinse 5-phosphosulfate (PAPS), which serves as the universal sulfonate donor for all cellular sulfonation reactions: a process of the transfer of a sulfonate group (SO3-1) from PAPS to appropriate acceptor molecules.

Hypothesis/Objective: We are examining the hypothesis that protein sulfonation induced by Snail is required for the induction of EMT and metastasis in breast cancer.

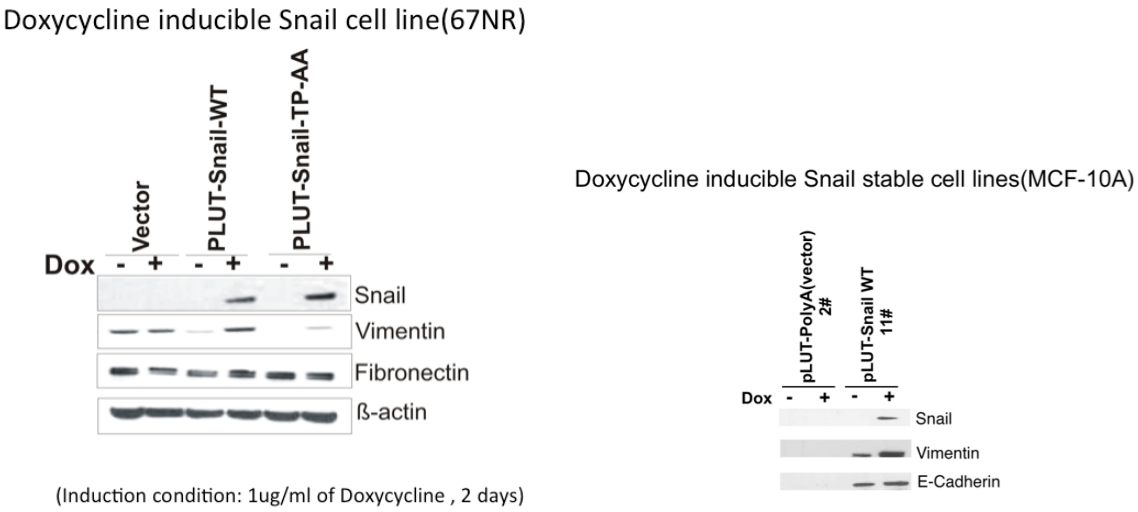
Specific Aims: We are performing experiments to: 1) test the hypothesis that PAPSS2 is required for maintaining the mesenchymal phenotype and invasive property of the breast cancer cells. 2) test the hypothesis that Snail-stimulated sulfonation of proteoglycans (PGs) are crucial for the induction of EMT and metastasis. **These experiments were approved in the original Proposal and Statement of Work and our experime ntalcourse has not deviated from this plan. Details of oure Progress are shown below.**

BODY:

We have made very good progress in the first year of support for this project. A key goal was to eatablish cell system wherein the expression and function of PAPSS2 and overall cellular sulfonation could be measured and correlated with both Snail levels/activity and correlate with metastatic potential of the cells. Our original goal was to characterize this in many established cell lines with already well defined Snail levels and metastatic potential. Over the course of the year , this strategy has proved problematic as the phenotypes and the expression levels in these well established, non-clonal cell lines are quite variable. In order to circumvent these problems, we have elected to jump to the strategy described in Aim 2. and derive clonal engineered cells lines using inducible Snail expression vectors. This has been well accomplished as described below:

A primary goal of this aim was to establish and inducible and reversible system to effect snail-mediated EMT in breast cancer cell lines using inducible plasmid or viral vectors and look at the induction on PAPSS2. This was critical in order to map the epigenetic marks placed in chromatin during both the induction and reversion of the EMT differentiation event and the role of sulfonation in these processes. Moreover, our specific strategy was to induce EMT only with a single transcription factor: Snail, in order to simplify the analysis of the marks Specifically induced by the holocomplex of Snail and its associated enzymatic machinery. We proposed to do this in the original grant proposal by making Snail fusion proteins to the Estrogen receptor hormone binding domain (HBD-TM) thus creating a tamoxifen responsive protein which could be turned on and off. We have had tremendous

positive experiences with this system in the past. Unfortunately, despite tremendous effort over the pats year, we have found that this system does not work with Snail family zinc finger proteins; The Snail-HBD fusions were poorly expressed in cells , the inductions were leaky and often unresponsive to hormone, and the regulation of both a Snail target gene and the EMT phenotype was variable and overall unsatisfactory. Thus about 6 months ago we moved to an entirely different inducible vector system, PLUT. This is based upon a TET-ON lentiviral system developed by my previous post-doctoral fellow Alexey Ivanov. He created a series of lentiviral vectors , which encode the TET –ON repressor', and a separate insert site for a candidate cDNA under the control of TET operators. The beauty of this system is that it is completely encoded in a single lentiviral vector, and thus separate TET-ON encoding vectors are not required. We created wt and mutant Snail vectors in this system, harvested high titer lentiviral stocks and used these to infect a myriad of breast cancer cells. Clonal derivatives form these cultures were isolated expanded and tested for induction of Snail protein by Doxycycline treatment, and for induction of the EMT phenotype. In the past year we have isolated and tested literally hundreds of clonal lines from separate transfections in order to identify novel founder lines. We thus now have beautiful set of stably transfected cell clones which have a low background level, but are robustly inducible for Snail after Dox treatment. An example is shown below (Fig 1) in both MCF10A cells and murine 67NR cells: Both wt Snail and mutant TP-AA Snail is robustly induced by Dox treatment of 67NR cells. The characteristic spindle morphology or EMT cells is seen in brightfield of wt but not mutant expressing cells. A similar result is seen in clonal cell lines derived from MCF10A cells. The induction of morphologic EMT is even more robust in these cells. Thus, a critical component of this Specific Aim has been accomplished, that is, derivation or inducible cell clones which respond exclusively to the Snail signal for EMT induction. These clones will be key to defining the epigenetic signatures, the histone modifications and the holocomplex of proteins assembled on promoters which comprise the EMT response and the regulation of PAPSS2 by Snail during EMT. Our preliminary results looking at PAPSS2 levels in these inducible cell lines indicates that indeed there is a good correlation between Snail levels and PAPSS2 levels. These expts. will be carried out in the remaining time of the award. Thus despite a few setbacks, we have largely accomplished this critical goal of AIM 2.



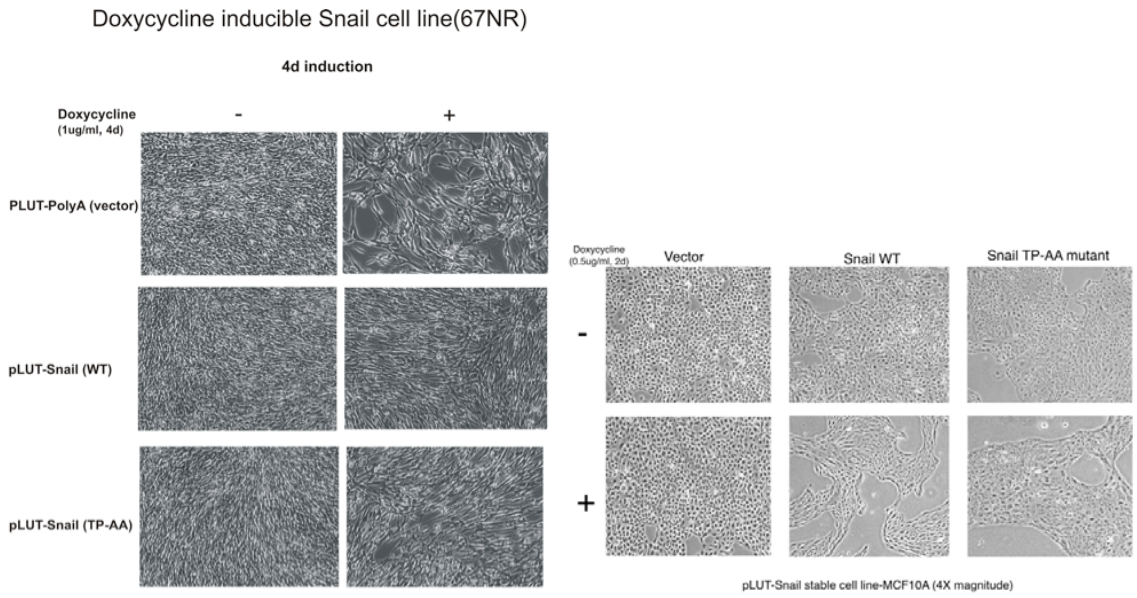


Figure 1: Creation of Doxcycline Inducible Snail Clonal Cell lines from MCF10A and 67NR cells.
Upper left, Western blot of Snail and other EMT markers in two clonal cell lines expressing either wt Snail or the Mutant TP-AA which destroys the T177 Phosphorylation site. Lower Left: Morphologic changes induced by Snail induction in 67NR cells. Lower right and Lower left: Similar inductions and analyses in clonal MCF10A cells lines.

A second major need for studying the PAPSS2 mediated cellular sulfonation pathways in EMT was the use of antibodies to the PAPSS1 and PAPSS2 enzymes such that abundance and activity could be detected following knockdown, overexpression and influence on sulfonation substrates. This has also turned out to be difficult as, only until recently have commercially available PAPSS2 antibodies. We have obtained these antibodie to both PAPSS1 and PAPSS2 . These abs turn out to be very clean reagents and detect full length PAPSS2 and PAPSS1 in the breast cancer cell lines MDA 231, MCF7 and MCF10A as shown in the western blot below (Fig 2). The levels of both enzymes are higher in the more highkly metastatic MCF7 and 231 cells compared to the MCF10A cells.

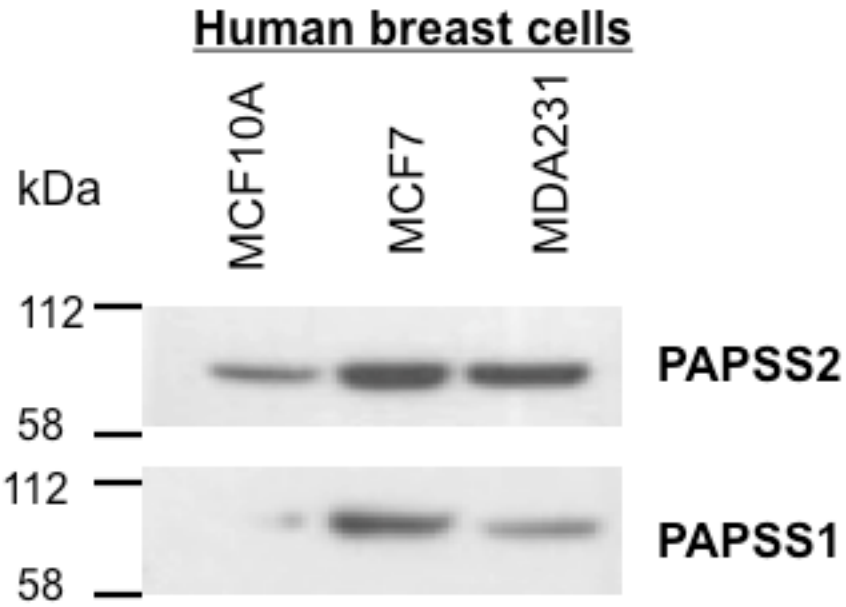


Figure 2 : Endogenous protein levels of PAPSS1, PAPSS2, and in human breast cells

A third major goals of the first year was to generate and characterize highly specific shRNA knockdown vectors that specifically target each PAP enzyme isoform. We have done so by evaluating a number of different vector designs and nucleotide sequences. In transient transfectins, these vectors have been shown to specifically target either one or the other isoforms. These vectors have now bee introduced into the cell lines shown above and stably knockdown clones are under selection. These clones will be analyzed for 1) knockdown of each enzyme by Western blot, 2) Influence on cell growth and motility , 3) effect on the metastatic phenotype. All of these experiments are in the original plan of the proposal and were scheduled to be accomplished in Year 2. We are on track for accomplishing this in year 2. In summary , in spite of some difficulties with vectors and reagents, we have made very good progress and are on track for competing the aims of the grant.

We are on track with the original Plan submitted in the SOW and there are no deviations of research strategy to report at this time,

KEY RESEARCH ACCOMPLISHMENTS: YEAR 1

1. Creation Tetracycline inducible WT Snail and mutant Snail vectors for expression in cell lines
2. Creation of stable cell lines in MCF10A cells and 67NR cells via a lentiviral expression system.
3. Demonstration MCF-10A cells undergo dramatic morphologic EMT in the presence of WT Snail expression, with the downregulation of the Cell adhesion molecule E-cadherin and the upregulation of the mesenchymal markers vimentin and fibronectin.
4. Characterization of antibodies to PAPSS1 and PAPSS2 enzymes.
5. Demonstration that bot h enzymes are expressed in Human breast cancer cell lin es awith a positive correlation to Snail levels
6. Derivation and characterization of shRNA knockdown vectors for PAPSS1 and PAPSS2.
7. Isolation or stably transfected clonal cell lines which have Knockdown of either PAPS S1 or PAPSS2

REPORTABLE OUTCOMES: None

CONCLUSION: In summary we are highly motivated to describe this completely novel cellular sulfonation pathway controlled by Snail through activation of gene expression to regulate EMT and metastasis in breast cancer cells. Our preliminary studies have shown that Snail induces the key enzyme in the sulfonation pathway called phosphoadenosinse phosphosulfate synthase 2 (PAPSS2) in breast cancer cells. PAPSS2 catalyzes the synthesis of PAPS, the only sulfate donor for all sulfonation reaction in cells. Strikingly, inactivation of this enzyme in the highly aggressive, mesenchymal-like, metastatic human breast cancer cells leads to profound acquisition of the epithelial phenotype, suggesting a Snail stimulated posttranslational modification of proteins and other biomolecules is critical in the regulation of EMT and metastasis. However, virtually nothing is known of this sulfonation pathway in the context of the transcriptome regulated by Snail in tumors. The next year of research funded by this grant should produce some striking discoveries in this pathway. We recommend no changes in strategy or approach as everything sis working and we have al systems and reagents in hand to answer the important questions.

REFERENCES: None

APPENDICES: None